On the Conformation of Caseins. Optical Rotatory Properties*

T. T. Herskovits†

ABSTRACT: The optical rotatory properties of the whole casein of cow's milk and the three major casein components, α_{s1}-, β-, and κ-casein, have been examined in both aqueous and organic solvents. Analysis of the dispersion data by means of the Moffitt–Yang, Drude, and Shechter and Blout equations and calibration constants, together with the analysis of the trough values of the 233-mμ Cotton effects, indicate very low α-helix content and a low degree of structural organization of the major casein components. Around neutral pH, the caseins, including whole casein, are characterized by low Moffitt b_0 parameters ranging from approximately -30 to $+30$, and very low a_0 values ranging -510 to -780; the Drude λ_c values range from 211 to 223 mμ. The Shechter and Blout $A(\alpha,\rho)_{193}$ and $A(\alpha,\rho)_{225}$ parameters are found to vary from -180 to -590 and -265 to -410, respectively, and the mean residue rotations at 233 mμ range from about -2800 to -3800. These findings tend to rule out α-helical organization as a basic feature of casein aggregates or micelles in aqueous media. It is also found that an appreciable degree of α-helix formation can be produced by dissolving the caseins in structure-forming solvents such as acidic methanol or 2-chloroethanol.

In the past decade considerable effort has been directed toward the isolation and characterization of the caseins and various casein components of milk (see the reviews of Lindqvist, 1963; Timasheff, 1964; Gordon and Whittier, 1965). However, because of the complex nature of the stoichiometry of casein–casein interactions in the neutral pH region, relatively little work has been done concerning the conformation of the caseins of their native state. The light scattering studies of Krescheck *et al.* (1964) and Krescheck (1965) have suggested that α_{s1}- and κ-casein in neutral solutions exist in the form of random coils. Payens and van Markwijk (1963) have concluded on the basis of ultracentrifugal and viscosity studies on β-casein that this casein component in neutral salt solutions behaves hydrodynamically as a rod-like aggregate or a coiled polymeric aggregate. Recent studies of Swaisgood and Timasheff (to be published), in this laboratory, have suggested that α_{s1}-casein in the neutral and slightly alkaline pH region also exists in the form of aggregates, having average molecular weights which correspond to trimers to pentamers.

Earlier reports of the sodium D rotations and Drude constants of some of the casein components (Hipp *et al.*, 1952; Jirgensons, 1958a,b; Herskovits, 1964; Herskovits and Mescanti, 1965), together with the present study, have suggested that the caseins possess a random conformation similar to the disorganized structure of denatured globular proteins. In addition to a detailed study of the optical rotatory properties of the caseins in aqueous media, the present work also reports the properties of the caseins in 8 M urea and 2-chloroethanol, and in the case of α_{s1}-casein, in a number of other organic solvents.[1]

Experimental Section

Materials. Whole casein and the genetic variants of α_{s1}-casein and β-casein A were generously provided by Dr. M. P. Thompson. The preparation of these proteins from the milk of single homozygous cows has been described by Thompson and Kiddy (1964) and Thompson and Pepper (1964). The B variant of β-casein

* From the Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Philadelphia, Pennsylvania 19118. *Received August 24, 1965; revised November 23, 1965.* Presented in part at the meeting of the American Chemical Society, Denver, Colo., Jan 1964.

† Present address: Department of Chemistry, Fordham Univ., The Bronx, New York.

[1] While this paper was in preparation Kresheck (1965) reported the rotatory dispersion parameters of pooled whole casein and β-casein. While the author's (Table I) Drude and Moffitt parameters are somewhat different from the Kresheck's (*i.e.*, λ_c = 233 and 212 mμ, a_0 = -455 and -530, and b_0 = -97 and -53 for whole casein and β-casein in pH 6.5, $\Gamma/2$ = 0.1 phosphate buffer, respectively), their conclusions are essentially the same; namely, that the caseins are largely devoid of α-helical organization.

was a gift of Dr. R. F. Peterson; this sample was prepared by the urea fractionation method of Hipp *et al.* (1952). The samples of κ-casein, wheat whole gluten and glutenin were generously provided by Dr. J. H. Woychik, while the calfskin collagen was a gift of Dr. L. D. Kahn. Poly-L-proline (Mann Research Laboratories) and zein were commercial products.[2] The organic reagents and solvents employed were analytical grade or of the purest grade commercially available.

Methods. The preparation of protein solutions in aqueous and organic solvents has been described (Herskovits, 1965). When necessary, protein solutions were clarified by centrifugation in a Spinco Model L preparative centrifuge or by filtration through an ultrafine sintered-glass filter. Protein concentrations were determined gravimetrically or spectrophotometrically by use of a Zeiss Model PMQ II spectrophotometer.

The following values of the absorptivity, $A^{1\%}_{1cm}$, at 280 mμ were employed to calculate the concentration of aqueous casein solutions: whole casein = 10.0, α_{s1}-casein = 10.1 (Thompson and Kiddy, 1964), β-casein = 4.6 (Thompson and Pepper, 1964), κ-casein = 12.2 (Zittle and Custer, 1963). The concentrations of whole gluten, glutenin, zein, collagen, and poly-L-proline solutions were based on dry-weight measurements. Before dissolving, these substances were dried overnight in a vacuum oven at 50–70°, transferred to and cooled in a desiccator, and then rapidly weighed. The concentrations of the protein solutions employed ranged from 0.008 to 0.5 g/100 ml.

Optical rotatory dispersion measurements in the visible and near-ultraviolet region (578–313 mμ) were made in a Rudolph Model 200S spectropolarimeter, employing both mercury and xenon arc lamps as sources of radiation. Measurements were made in a room maintained at 25 ± 1°. Low-temperature measurements were carried out by use of a specially designed 10-cm all-quartz jacketed cell. The desired temperature of the casein solutions was maintained by circulating water from a thermoregulated bath through both the cell compartment of the instrument and the jacket of the cell. Because of the relatively low rates of dissociation of β-casein aggregates (Payens and van Markwijk, 1963), before the low-temperature experiments β-casein solutions were equilibrated for 24 hr at 0–1°. Measurements in the ultraviolet region (220–300 mμ) were made in a Cary Model 60 spectropolarimeter. The instrument was calibrated with sucrose solutions by Drs. Tomimatsu and Gaffield, following the procedure of Yang and Samejima (1963).

Treatment of Rotatory Dispersion Data. The rotatory properties of the protein solutions in the 313–578 mμ region were interpreted by means of the Moffitt–Yang equation

$$[R']_\lambda = [3M_0/100(n^2 + 2)][\alpha]_\lambda = a_0\lambda_0^2/(\lambda^2 - \lambda_0^2) + b_0\lambda_0^4/(\lambda^2 - \lambda_0^2)^2 \quad (1)$$

[2] Mention of specific manufacturers or products does not imply endorsement by the U. S. Department of Agriculture to the possible exclusion of others not mentioned.

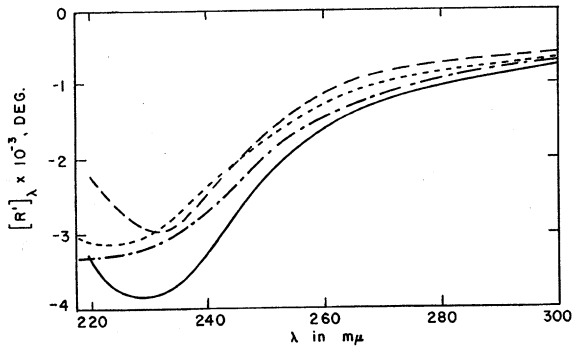

FIGURE 1: Optical rotatory dispersion of caseins in aqueous 0.025 M NaCl, pH 6.0–7.7. ———, whole casein II; — — —, α_{s1}-casein C; —·—·, β-casein B; -------, κ-casein.

the one-term Drude equation

$$[\alpha]_\lambda = A/(\lambda^2 - \lambda_c^2) \quad (2)$$

and the Shechter and Blout (1964a) modification of the two-term Drude equation

$$[R']_\lambda = A(\alpha,\rho)_{193}\lambda_{193}^2/(\lambda^2 - \lambda_{193}^2) + A(\alpha,\rho)_{225}\lambda_{225}^2/(\lambda^2 - \lambda_{225}^2) \quad (3)$$

The a_0 and b_0 parameters in eq (1) are evaluated from the intercept and slope, respectively, of a plot of $[R']_\lambda \cdot (\lambda^2 - \lambda_0^2)$ *vs.* $1/(\lambda^2 - \lambda_0^2)$ plots. The constants A and λ_c are similarly obtained from the intercept and slope of a plot of $[\alpha]_\lambda\lambda^2$ *vs.* $[\alpha]_\lambda$. The $A(\alpha,\rho)_{193}$ and $A(\alpha,\rho)_{225}$ parameters may be obtained from a plot of $[R']_\lambda \cdot (\lambda^2 - \lambda_{193}^2)/\lambda_{193}^2$ *vs.* $\lambda_{225}^2/(\lambda^2 - \lambda_{225}^2)$. The slope and intercept of such a linear plot are equal to $A(\alpha,\rho)_{225} \cdot [(\lambda_{225}^2 - \lambda_{193}^2)/\lambda_{193}^2]$ and $A(\alpha,\rho)_{193} + A(\alpha,\rho)_{225}(\lambda_{225}^2/\lambda_{193}^2)$, respectively, yielding $A(\alpha,\rho)_{225}$ from the slope and $A(\alpha,\rho)_{193}$ from the intercept. In conjunction with eq (1) and (3) the following mean residue molecular weights, M_0, were employed: whole casein = 119, α_{s1}-casein = 119.5, β-casein = 119.1, κ-casein = 118, whole gluten and glutenin = 113, zein = 92.2, calfskin collagen = 91.3, poly-L-proline = 97.1. The water, urea, 2-chloroethanol, and formic acid data were corrected for the dispersion of refractive index. The dispersion of refractive indices of methanol and ethylene glycol have not been reported. However, the effect of this correction on Moffitt parameters of the former solvents is only of the order of 1–2%, and, therefore, no corrections were applied to the latter two solvents. The Moffitt parameter and the Shechter and Blout parameters were calculated by use of a Fortran program, with an IBM 1620 computer.

Results

Optical Rotatory Properties of the Caseins in Neutral Aqueous Media. The optical rotatory dispersion data of

TABLE I: Optical Rotatory Parameters of Caseins.[a,b]

Protein	$-A \times 10^{-6}$	λ_c (mμ)	a_0	b_0	$A(\alpha,\rho)_{193}$	$A(\alpha,\rho)_{225}$	$[R']_{233}$
Whole Casein I	37.5	211	−780	+30	−590	−265	...
Whole Casein II	34.0	217	−720	−25	−330	−400	−3800
α_{s1}-Casein B	23.7	223	−515	−15	−240	−380	−3400
α_{s1}-Casein C	24.0	221	−510	−30	−180	−320	−2850
β-Casein A	33.6	218	−705	−30	−300	−410	−3090
β-Casein B	35.4	215	−735	−20	−515	−275	−3110
κ-Casein	36.1	218	−765	−10	−415	−375	−2820

[a] With the exception of $[R']_{233}$ the dispersion parameters are based on measurements in the 313–578 mμ region; the $[R']_{233}$ values are based on measurements in the 220–300 mμ region (Figure 1). [b] In KCl–phosphate buffer or NaCl, Γ/2 = 0.1, pH 7.0–7.4 except for $[R']_{233}$ which was obtained in 0.025 M NaCl, pH 6.0–7.7.

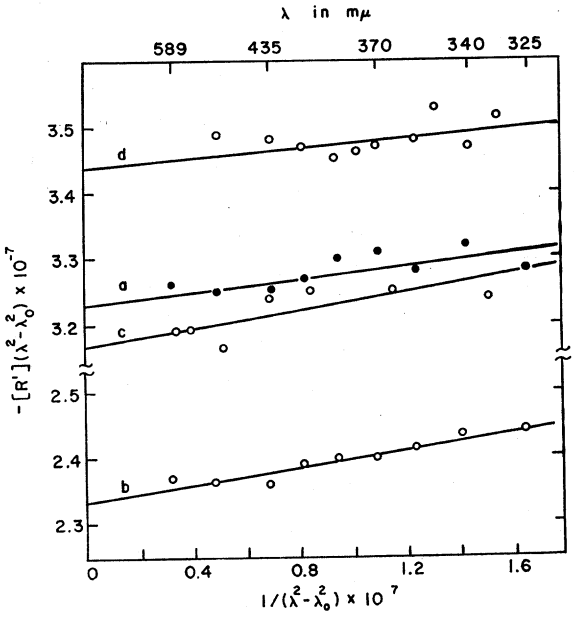

FIGURE 2: Moffitt–Yang plots of caseins in KCl–phosphate, Γ/2 = 0.1, pH 7.0. Curve a, whole casein II; curve b, α_{s1}-casein C; curve c, β-casein A; curve d, κ-casein. The lines were computed by the method of least squares.

whole casein and the three casein components in 0.025 M NaCl obtained in the 220–300 mμ region are shown in Figure 1. The troughs of the 225-mμ Cotton effects are relatively small compared to the troughs observed with most helical polypeptides and proteins (Simmons and Blout, 1960; Simmons *et al.*, 1961; Yang and Samejima, 1963). The optical rotatory power of the caseins below the trough was too low to allow reliable measurements below about 220 mμ. Figure 2 shows the Moffitt–Yang representation of the dispersion properties of the caseins in the 313–578 mμ region. The dispersion constants of the caseins in neutral aqueous media, based on eq (1), (2), and (3) and the data of Figure 1, are summarized and compared in Table I. Estimates of the helix content, given in Table II, were based on the following empirical equations (Yang, 1961; Urnes and Doty, 1961; Shechter and Blout, 1964a,b; Shechter *et al.*, 1964)

$$\% \text{ helix } (\lambda_c) = (\lambda_c - 212)/1.40 \quad (4$$

$$\% \text{ helix } (b_0) = -b_0/6.30 \quad (5)$$

$$\% \text{ helix } (193) = [A(\alpha,\rho)_{193} + 750]/36.5 \quad (6)$$

$$\% \text{ helix } (225) = -[A(\alpha,\rho)_{225} + 60]/19.9 \quad (7)$$

and on the trough value, $[R']_{233}$, for the 225-mμ Cotton effect.

$$\% \text{ helix } [R']_{233} = -([R']_{233} + 1900)/138 \quad (8)$$

For proteins in most organic solvents, eq (6) and (7) have to be replaced by eq (9) and (10), which have somewhat different empirical constants.

$$\% \text{ helix } (193) = [A(\alpha,\rho)_{193} + 600]/36.2 \quad (9)$$

$$\% \text{ helix } (225) = -A(\alpha,\rho)_{225}/19.0 \quad (10)$$

The constants employed in eq (8) are the average values obtained on poly-L-glutamic acid in the helical and random conformation by three groups of workers (Blout *et al.*, 1962; Yang and Samejima, 1963; Breslow *et al.*, 1965).

While there are serious questions about the precise numerical values of these calibration constants (Yang, 1965), including the constants of eq (5), the literature of the subject need not be reviewed here, since it is fairly apparent from the data of Table II that the individual caseins, as well as the complexes found in whole casein solutions (Waugh, 1958; Noble and

TABLE II: Estimates of Helix Content of Caseins in Aqueous and 8 M Urea Solutions.[a]

Solvent and Protein	% Helix				
	Yang Eq (4)	Moffitt Eq (5)	Shechter–Blout Eq (6)	Shechter–Blout Eq (7)	$[R']_{233}$ Eq (8)
Aqueous[b]					
Whole casein	2	0	8	14	14
α_{s1}-Casein	5	4	15	12	9
β-Casein	3	4	9	14	9
κ-Casein	4	2	9	16	7
8 M Urea					
Whole Casein I	1	0	2	14	..
α_{s1}-Casein C	4	0	11	11	..
β-Casein A	0	0	5	13	..
κ-Casein	1	0	3	15	..

[a] Estimates based on data of Tables I and III. [b] Based on average values of the dispersion parameters of samples I and II of whole casein and the two genetic variants of α_{s1}- and β-casein.

TABLE III: Optical Rotatory Parameters of Caseins in 8 M Urea.[a]

Protein	Γ/2	pH	$-A \times 10^{-6}$	λ_c (mμ)	a_0	b_0	$A(\alpha,\rho)_{193}$	$A(\alpha,\rho)_{225}$
Whole casein, I	0.1	7.5	45.7	213	−925	+30	−675	−335
α_{s1}-Casein C	0.03	3.3	30.5	217	−620	0	−365	−285
β-Casein A	0.1	7.4	41.4	212	−835	+15	−565	−325
κ-Casein	0.1	7.4	46.0	214	−925	+20	−650	−359

[a] Parameters are based on the dispersion data in the 313–578 mμ region.

Waugh, 1965) are largely devoid of helical organization.

On examining the data of Table I and Figures 1 and 2, it is worth noting that the values of the rotatory dispersion parameters of whole casein are approximately equal to average values of the constituent major casein fractions,[3] α_{s1}-, β-, and κ-casein, if allowance is made for the fact that the formation of aggregates, such as the α_s–κ and β– complexes in neutral salt solutions (Waugh, 1958; Payens and van Markwijk, 1963), can produce minor changes in the dispersion parameters. Changes in the Moffitt a_0 parameter, accompanying the association of β-lactoglobulin, have been observed (Herskovits *et al.*, 1964). The fact that the helical content of whole casein, as reflected by the Moffitt and Shechter and Blout parameters, is very low mitigates against the possibility that the apparently nonhelical or low helical character of the individual casein components is an artifact of the procedure employed in their isolation. In contrast to the isolation of the individual casein fractions, which involve urea fractionation and treatment with alcohol (Hipp *et al.*, 1952; Thompson and Kiddy. 1964; and Thompson and Pepper, 1964), whole casein is isolated by a fairly mild procedure which only involves the exposure of the milk proteins to pH 4.6–4.8.

Effects of Temperature and Denaturing Solvents. The urea denaturation of most globular proteins is accompanied by a fairly pronounced change in the Drude and Moffitt–Yang parameters. Most denatured proteins in concentrated urea or aqueous solutions have λ_c values in the neighborhood of 210–220, $a_0 = -600$ to -800, and $b_0 = 0$ to -50 (Urnes and Doty, 1961). Comparison of the data of Tables I and III indicate that the changes in these parameters of the caseins are relatively small, supporting the idea that the caseins have a largely disorganized, random-polypeptide conformation.

Such a conformation should be characterized by a fair degree of flexibility of the protein chain (Kresheck *et al.*, 1964; Payens and van Markwijk, 1963). That this is in fact the case is suggested by effect of temperature on the rotatory dispersion parameters. As is shown by the data of Table IV, lowering the temperature from 25 to 1.5° causes a small but definite increase in the b_0 values of the three caseins. The rotatory dispersion

[3] Whole casein consists of approximately 55% α_s-, 25–30% β-, 15% κ-casein, and a few per cent of additional minor components designated as λ- and γ-caseins (Lindqvist, 1963).

TABLE IV: Effect of Temperature on the Optical Rotatory Parameters of Caseins.[a]

Protein[b]	Temp, °C	$-A \times 10^{-6}$	λ_c (mμ)	a_0	b_0	% Helix Eq (5)
α_{s1}-Casein C	25	24.8	218	−520	−35	6
	1.6	27.2	216	−570	−20	4
β-Casein A	25	33.6	218	−705	−30	5
	1.5	40.8	214	−855	−5	1
κ-Casein	25	35.5	216	−735	−30	5
	1.5	39.0	215	−815	−5	1

[a] Parameters are based on the dispersion data in the 313–578 mμ region. [b] In KCl–phosphate, Γ/2 = 0.1, pH 7.0.

TABLE V: Rotatory Dispersion Parameters and Proline and Hydroxyproline Content of Caseins and Related Proteins in 2-Chloroethanol.[a]

Protein	a_0	b_0	$A(\alpha,\rho)_{193}$	$A(\alpha,\rho)_{225}$	Mole % Proline and Hydroxyproline
α_{s1}-Casein C	+5	−300	+1250	−915	8.5
α_{s1}-Casein B[b]	+5	−290	+1215	−890	8.8
Zein	−75	−215	+845	−685	10.0
κ-Casein[b]	−160	−355	+1385	−1160	10.8
Whole casein I	−130	−370	+1460	−1190	11.5
Glutenin[b]	−170	−190	+600	−610	13.5
Whole gluten	−235	−180	+605	−655	16.2
β-Casein A	−220	−225	+800	−785	16.8
Calfskin collagen[b]	−245	−80	+50	−260	23.2
Poly-L-proline	−1950	+125	−1745	−460	100.0

[a] The Moffitt–Yang and Shechter and Blout parameters are based on the dispersion data in the 313–578 mμ region. [b] In the presence of 0.01 M HCl.

studies on β-lactoglobulin (Herskovits *et al.*, 1964), myoglobin, and apomyoglobin (Harrison and Blout, 1965) indicate that no such changes accompany the cooling of aqueous solutions of these more rigid globular proteins.

Cooling the casein solutions is also accompanied by a decrease in λ_c and a_0. It is worth noting that the greatest change in these parameters accompanies the cooling of β-casein solutions. This may be a reflection of both the change in conformation (helical content) plus the effect of dissociation of the aggregates of this protein as the temperature is lowered below 6° (Payens and van Markwijk, 1963). At room temperature β-casein exists in the form of stoichiometric aggregates consisting of about 30 monomer units (Payens and van Markwijk, 1963). α_{s1}-Casein and κ-casein associate to a much less extent and the aggregates of these two caseins are known not to dissociate completely as the temperature is lowered (Sullivan *et al.*, 1955; Waugh, 1958). In this connection it is perhaps significant that β-casein has the highest proline content of the three casein fractions investigated (Table V). It has been suggested that high proline content in certain proteins may lead to the formation of a collagen-like, so-called poly-L-proline II type of fold (Harrington and Sela, 1958). The possibility that some of the proline-rich segments of β-casein at low temperature may, in fact, assume a polyproline II-like conformation should be considered. That only a small fraction of the polypeptide chain can assume this conformation is suggested by the fact that the change in the Moffitt a_0 parameter for β-casein, upon cooling, is −150°, whereas the corresponding change accompanying the cooling of gelatin is of the order of −1000 to −1200°. Dispersion measurements on calfskin collagen in pH 3.7, Γ/2 = 0.13, citrate buffer gave $a_0 = -2020°$ and $b_0 = +260°$.

TABLE VI: Optical Rotatory Parameters of α_{s1}-Casein in Random Coil Forming and Helix-Promoting Solvents.[a]

Solvent	$-A \times 10^{-6}$	λ_c (mμ)	a_0	b_0	$A(\alpha,\rho)_{193}$	$A(\alpha,\rho)_{225}$
Water, 0.03 M Cl^-, pH 1.7	22.8	228	−480	−30	−155	−315
Water, 0.03 M Cl^-, pH 7.4	24.8	223	−530	−45	−135	−370
Water, 0.03 M Cl^-, pH 11.6	29.4	223	−650	+25	−500	−210
97% Formic acid	21.8	224	−450	−40	−115	−320
8 M Urea, 0.03 M Cl^-, pH 3.3	30.5	217	−620	0	−365	−280
Ethylene glycol	15.2	248	−295	−140	+415	−570
2-Chloroethanol	*b*	*b*	+5	−300	+1250	−915
Methanol, 0.01 M HCl	*b*	*b*	−55	−345	+1420	−1095

[a] Parameters based on the dispersion data in the 313–578 mμ region. [b] Dispersion is anomalous (*i.e.*, the $[\alpha]_\lambda\lambda^2$ *vs.* $[\alpha]_\lambda$ plot is not linear).

Upon denaturation in 8 M urea these changed to −900° and +60°, respectively.[4] Similar observations were made by von Hippel and Wong (1963) on ichthyocol collagen.

TABLE VII: Estimates of Helix Content of the Caseins in Helix-Promoting Solvents.[a]

Protein and Solvent	% Helix		
	Moffitt Eq (5)	Shecter and Blout Eq (9)	Shecter and Blout Eq (10)
α_{s1}-Casein C			
Ethylene glycol	22	28	30
Methanol, 0.01 M HCl	55	59[b]	52[b]
2-Chloroethanol	48	51	48
β-Casein A			
2-Chloroethanol	36	39	41
κ-Casein			
2-Chloroethanol, 0.01 M HCl	56	55	61
Whole casein I			
2-Chloroethanol	59	57	63

[a] Estimates based on data of Tables V and VI. [b] The methanol values are based on eq (6) and (7). These equations are found to be more appropriate for more polar, hydrogen-bonding solvents (Schecter and Blout, 1964,b).

[4] In addition to having a positive Moffitt b_0 parameter, collagen and polyproline II also exhibit strongly negative Cotton effects, with minima at 210–220 mμ (*e.g.*, $[R']_{208} \sim -35{,}000$ for calfskin collagen (Blout *et al.*, 1963). Based on b_0, polyproline II-like regions in proteins would thus lead to fortuitously low estimates of helix content, while calculations based on the trough value of the 225-mμ Cotton effect would lead to high estimates of helix content. The close agreement between these two estimates of helix content for the caseins (Table II) suggests that such regions must be largely absent in the case of these proteins at room temperature.

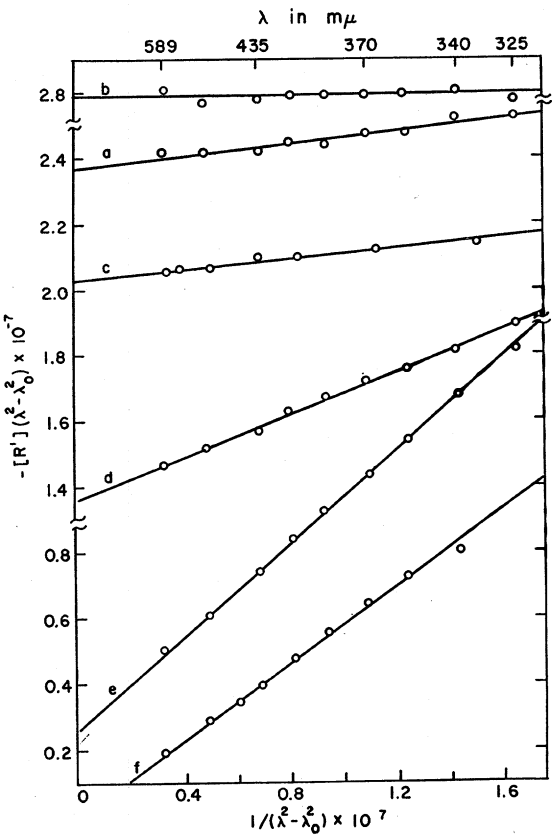

FIGURE 3: Moffitt–Yang plots of α_{s1}-casein C in aqueous and organic solvents. Curve a, water, 0.03 M Cl^-, pH 7.4; curve b, 8 M urea, 0.03 M Cl^-, pH 3.3; curve c, 97% formic acid; curve d, ethylene glycol; curve e, methanol, 0.01 M HCl; curve f, 2-chloroethanol. Lines were computed by the method of least squares.

The effect of a second random coil promoting solvent, formic acid, on the rotatory properties of α_{s1}-casein is also relatively small. On the other hand, the solvents ethylene glycol, 2-chloroethanol, and methanol have a pronounced effect on the rotatory properties of

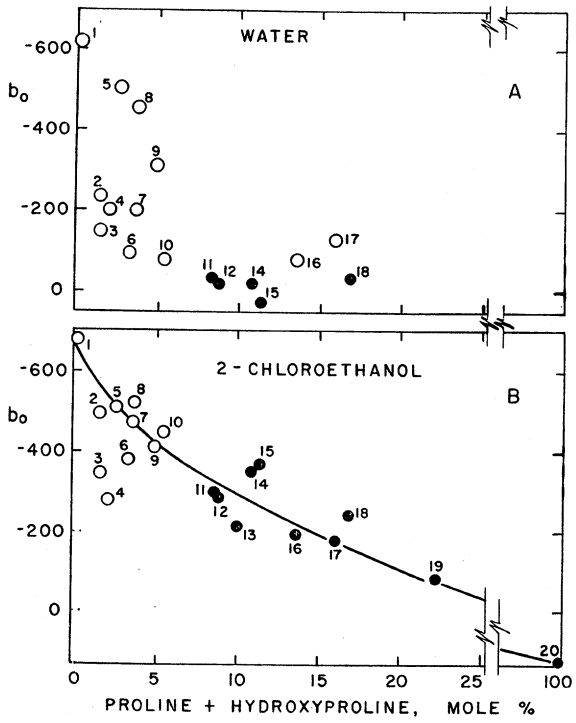

FIGURE 4: Variation of the Moffitt b_0 parameter as a function of proline and hydroxyproline content of proteins; (A) in aqueous solution; (B) in 2-chloroethanol. Numbers designate the following proteins and polypeptides: 1, tropomyosin; 2, α-lactalbumin; 3, lysozyme; 4, insulin; 5, myoglobin and globin M (in 2-chloroethanol); 6, ribonuclease; 7, ovalbumin; 8, hemoglobin and globin H (in 2-chloroethanol); 9, bovine serum albumin; 10, β-lactoglobulin; 11, α_{s1}-casein C; 12, α_{s1}-casein B; 13, zein; 14, κ-casein; 15, whole casein I; 16, glutenin; 17, whole gluten; 18, β-casein A; 19, calfskin collagen; 20, poly-L-proline. Filled circles refer to data of Tables I, V, and VI; open circles refer to data taken from Urnes and Doty (1961), Beychok and Blout (1961), Herskovits and Mescanti (1965), and Wu and Cluskey (1965).

the caseins (Tables V-VII and Figure 3). Judging by the changes in the Moffitt and the Schechter and Blout parameters, it appears that the helical content of the caseins can be increased to as much as 50–60% (Tables II and VII).

Effects of Variations in the Chemical Composition of the Caseins. There are three known genetic variants of both α_{s1}- and β-caseins (Thompson and Kiddy, 1964; Thompson and Pepper, 1964) and recently genetic polymorphism has also been found in κ-casein (Woychik, 1964). The three polymorphs of α_{s1}- and β-caseins are designated A, B, and C (Thompson and Kiddy, 1964). As in the case of the β-lactoglobulins (Herskovits *et al.*, 1964; Townend *et al.*, 1964) genetic polymorphism, that is the replacement of substitution of individual amino acids as a result of genetic mutations, has little or no effect on their rotatory dispersion properties and, therefore, on their secondary structure This is shown by the data of Table I, where the rotatory dispersion properties of α_{s1}-casein B and C and β-casein A and B are compared.

Effect of Proline Content. Figure 4 presents a correlation between the Moffitt b_0 parameter, which reflects the helical content of proteins, and the proline and hydroxyproline content of various proteins, including the caseins, in aqueous media and in 2-chloroethanol. The data represented by open circles were taken from the literature, while the filled circles represent data of this study (Table I and V). The data obtained in aqueous media on the caseins are in agreement with the conclusion of Szent-György and Cohen (1957) concerning the essentially nonhelical character of proline-rich proteins. As is expected, even in the strongly helix-promoting solvent, 2-chloroethanol, the helical content of the caseins as well as the other proline-rich proteins, zein, gluten, and glutenin, is lower than that of proteins that contain less proline.

Discussion

It has been recognized for some time that the proline ring cannot be accommodated into the α-helical fold in proteins. Szent-György and Cohen (1957) have concluded, from optical rotatory dispersion studies on various myosins and fibrinogen, that the disruptive influence of proline on the polypeptide chain is such that with about 8% or more proline statistically distributed through the protein the polypeptide backbone will be deformed into a random coil. The proline content of the caseins is 8.5–16.8 mole % (Table V); furthermore, other amino acids (valine, isoleucine, serine, threonine, and the cystine contained in κ-casein (Jollès *et al.*, 1962; Swaisgood and Brunner, 1963)) may also interfere with helix formation (Blout *et al.*, 1960; Davies, 1964). It is then not surprising that the caseins have little α-helical organization (Table II).[1]

The effect of temperature and organic solvents on the optical rotatory properties of the casein is consistent with the idea that these proteins have a disorganized structure, usually encountered in the case of denatured proteins. One would expect on the basis of Schellman's (1958a,b) studies on denatured proteins that the rotatory properties of proteins with little structural organization should respond to changes in temperature (Table IV). Moreover, as is also expected, urea and formic acid, two random coil forming solvents, have relatively little effect on the rotatory properties of the caseins (Table II). On the other hand, structure-forming solvents such as 2-chloroethanol and acidic methanol are found to promote a fairly high degree of α-helical folding in these proteins (Tables V, VI, and VII). The fact that the caseins in their native state are readily digested by proteolytic enzymes (Northrop *et al.*, 1948; Nomoto *et al.*, 1960) is also consistent with the lack of extensive secondary and tertiary structural organization of these proteins. Linderstrøm-Lang (1952) some time ago made the observation that globular proteins often are partially or even totally resistant

to enzymatic attack in their native state. The same proteins are readily digested by these enzymes upon denaturation.

Waugh (1958) has suggested on the basis of hydrodynamic and molecular weight information that the three main casein components, α_s-, β-, and κ-casein, are rod-like macromolecules with lengths of 150–215 A and approximate diameters of 16 A. The fact that κ-casein could interact with four times its weight of α_s-casein (Waugh and von Hippel, 1956) has led to the notion (Waugh, 1958) that the probable structure of the α_s–κ-casein complex was a rod-like side-to-side aggregate consisting of four peripherally located α_s-casein molecules and one centrally located, but slightly protruding κ-casein molecule. More recently, however, Zittle *et al.* (1962) and Waugh and Noble (1965) have found that α_s–κ-casein complexes may actually be stabilized at weight ratios far in excess of 1:4, suggesting that Waugh's earlier model of casein complexes may be an oversimplification (see also Noble and Waugh, 1965).

While the rotatory dispersion data obtained on whole casein (Tables I and II) are not actually inconsistent with Waugh's model of the α–κ-casein complex, from what is known about the effect of intermolecular association of proteins (Schellman, 1958a,b; Urnes and Doty, 1961; Herskovits *et al.*, 1964) one would be led to believe that the formation of ordered rod-like aggregates from random chains of the individual casein components should cause a greater change in the optical rotatory properties than is actually observed[5] (Table II and Figures 1 and 2). These observations, together with the recent discovery (Beeby, 1963; Swaisgood and Brunner, 1963; Swaisgood *et al.*, 1964) that κ-casein consists of two or three disulfide-bond-linked polypeptide chains and has a molecular weight of about 50,000 (Waugh assumed that κ-casein consists of a single polypeptide chain having a molecular weight of 16,000), will require a revision of Waugh's earlier model of the basic subunit of casein micelles in solution. Recently Shimmin and Hill (1964) have advanced, on the basis of electron microscopic studies, an approximately spherical α_s–κ–β-casein complex of about 300,000 mol wt as a possible model for the basic subunit of casein micelles.

[5] Assuming that upon monomer formation the a_0 values of α_{s1}- and κ-casein would change by about the same amount as β-casein (20%; Table IV), the a_0 value of a hypothetical mixture of the major casein components (55% α_{s1}-, 30% β-, and 15% κ-casein; Lindquist, 1963) in their monomeric state would be equal to -735. In comparison, the average a_0 value of whole casein aggregates is -750 (Table I). The a_0 values of most denatured proteins and polypeptides in the random-coil form range from -500 to -800 (Urnes and Doty, 1961). Differences between the other rotatory dispersion parameters (λ_c, b_0, $A(\alpha,\rho)_{193}$, $A(\alpha,\rho)_{225}$, and $[R']_{233}$) of the individual casein components and whole casein are also relatively insignificant (Table I and IV), suggesting no obvious structural differences between whole casein aggregates and their constituent casin components.

Acknowledgment

The author is grateful to Drs. M. Noelken, G. C. Nutting and M. P. Thompson for valuable discussions and criticism of the manuscript. He also wishes to thank Dr. W. Gaffield for performing the rotatory dispersion measurements in the ultraviolet region for him, Dr. Y. V. Wu for a prepublication copy of a manuscript, Mr. T. F. Kumosinski for the computer calculations and Miss L. Mescanti for expert technical assistance.

References

Beeby, R. (1963), *J. Dairy Res. 30*, 77.

Beychok, S., and Blout, E. R. (1961), *J. Mol. Biol. 3*, 769.

Blout, E. R., Carver, J. P., and Gross, J. (1963), *J. Am. Chem. Soc. 85*, 644.

Blout, E. R., deLozé, C., Bloom, S. M., and Fasman, G. D. (1960), *J. Am. Chem. Soc. 82*, 3787.

Blout, E. R., Schmier, I., and Simmons, N. S. (1962), *J. Am. Chem. Soc. 84*, 3193.

Breslow, E., Beychok, S., Hardman, K. D., and Gurd, F. R. N. (1965), *J. Biol. Chem. 240*, 304.

Davies, D. R. (1964), *J. Mol. Biol. 9*, 605.

Gordon, W. G., and Whittier, E. O. (1965), *in* Fundamentals of Dairy Chemistry, Webb, B. H., and Johnson, A. H., Ed., Westport, Conn., Avi Publishing Co., p 54.

Harrington, W. F., and Sela, M. (1958), *Biochim. Biophys. Acta 27*, 24.

Harrison, S. C., and Blout, E. R. (1965), *J. Biol. Chem. 240*, 299.

Herskovits, T. T. (1964), Abstracts, 146th National Meeting of the American Chemical Society, Denver, Colo., Jan, p 22A.

Herskovits, T. T. (1965), *J. Biol. Chem. 240*, 628.

Herskovits, T. T., and Mescanti, L. (1965), *J. Biol. Chem. 240*, 639.

Herskovits, T. T., Townend, R., and Timasheff, S. N. (1964), *J. Am. Chem. Soc. 86*, 4445.

Hipp, N. J., Groves, M. L., Custer, J. H., and McMeekin, T. L. (1952), *J. Dairy Sci. 35*, 272.

Jirgensons, B. (1958a), *Arch. Biochem. Biophys. 74*, 57.

Jirgensons, B. (1958b), *Arch. Biochem. Biophys. 74*, 70.

Jollès, P., Alais, C., and Jollès, J. (1962), *Arch. Biochem. Biophys. 98*, 56.

Kresheck, G. C. (1965), *Acta Chem. Scand. 19*, 375.

Kresheck, G. C., van Winkle, Q., and Gould, I. A. (1964), *J. Dairy Sci. 47*, 117.

Linderstrøm-Lang, K. U. (1952), Proteins and Enzymes, Stanford, Calif., Stanford Univ. Press, p 53.

Lindqvist, B. (1963), *Dairy Sci. Abstr. 25*, 257.

Noble, R. W., Jr., and Waugh, D. F. (1965), *J. Am. Chem. Soc. 87*, 2236.

Nomoto, M., Narahashi, Y., and Murakami, M. (1960), *J. Biochem. (Tokyo) 48*, 593.

Northrop, J. H., Kunitz, M., and Herriott, R. M. (1948), Crystalline Enzymes, 2nd ed, New York, N. Y., Columbia Univ. Press, p 120.

Payens, T. A. J., and van Markwijk, B. W. (1963), *Biochim. Biophys. Acta 71*, 517.
Schellman, J. A. (1958a), *Comp. Rend. Lab. Carlsberg Sér. Chim. 30*, 395.
Schellman, J. A. (1958b), *Comp. Rend. Lab. Carlsberg Sér. Chim. 30*, 439.
Shechter, E., and Blout, E. R. (1964a), *Proc. Natl. Acad. Sci. U. S. 51*, 695.
Shechter, E., and Blout, E. R. (1964b), *Proc. Natl. Acad. Sci. U. S. 51*, 794.
Shechter, E., Carver, J. P., and Blout, E. R. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 1029.
Shimmin, P. D., and Hill, R. D. (1964), *J. Dairy Res. 31*, 121.
Simmons, N. S., and Blout, E. R. (1960), *Biophys. J. 1*, 55.
Simmons, N. S., Cohn, C., Szent-Györgyi, A. G., Wetlaufer, D. B., and Blout, E. R. (1961), *J. Am. Chem. Soc. 83*, 4766.
Sullivan, R. A., Fitzpatrick, M. M., Stanton, E. K., Annino, R., Kissel, G., and Palermiti, F. (1955), *Arch. Biochem. Biophys. 55*, 455.
Swaisgood, H. E., and Brunner, J. R. (1963), *Biochem. Biophys. Res. Commun. 12*, 148.
Swaisgood, H. E., Brunner, J. R., and Lillevik, H. A. (1964), *Biochemistry 3*, 1616.
Szent-Györgyi, A. G., and Cohen, C. (1957), *Science 126*, 697.
Thompson, M. P., and Kiddy, C. A. (1964), *J. Dairy Sci. 47*, 626.
Thompson, M. P., and Pepper, L. (1964), *J. Dairy Sci. 47*, 633.
Timasheff, S. N. (1964), *in* Symposium on Foods: Proteins and Their Reactions, Schultz, H. W., and Anglemier, A. F., Ed., Westport, Conn., Avi Publishing Co., p 179.
Townend, R., Herskovits, T. T., Swaisgood, H. E., and Timasheff, S. N. (1964), *J. Biol. Chem. 239*, 4196.
Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.
von Hippel, P. H., and Wong, K.-Y. (1963), *Biochemistry 2*, 1387.
Waugh, D. F. (1958), *Discussions Faraday Soc. 25*, 186.
Waugh, D. F., and Noble, R. W., Jr. (1965), *J. Am. Chem. Soc. 87*, 2246.
Waugh, D. F., and von Hippel, P. H. (1956), *J. Am. Chem. Soc. 78*, 4576.
Woychik, J. H. (1964), *Biochem. Biophys. Res. Commun. 16*, 267.
Wu, Y. V., and Cluskey, J. E. (1965), *Arch. Biochem. Biophys. 112*, 32.
Yang, J. T. (1961), *Tetrahedron 13*, 143.
Yang, J. T. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 438.
Yang, J. T., and Samejima, T. (1963), *J. Biol. Chem. 238*, 3262.
Zittle, C. A., and Custer, J. H. (1963), *J. Dairy Sci. 46*, 1183.
Zittle, C. A., Thompson, M. P., Custer, J. H., and Cerbulis, J. (1962), *J. Dairy Sci. 45*, 807.

TABLE VI: Optical Rotatory Parameters of α_{s1}-Casein in Random Coil Forming and Helix-Promoting Solvents.[a]

Solvent	$-A \times 10^{-6}$	λ_c (mμ)	a_0	b_0	$A(\alpha,\rho)_{193}$	$A(\alpha,\rho)_{225}$
Water, 0.03 M Cl^-, pH 1.7	22.8	228	−480	−30	−155	−315
Water, 0.03 M Cl^-, pH 7.4	24.8	223	−530	−45	−135	−370
Water, 0.03 M Cl^-, pH 11.6	29.4	223	−650	+25	−500	−210
97% Formic acid	21.8	224	−450	−40	−115	−320
8 M Urea, 0.03 M Cl^-, pH 3.3	30.5	217	−620	0	−365	−280
Ethylene glycol	15.2	248	−295	−140	+415	−570
2-Chloroethanol	*b*	*b*	+5	−300	+1250	−915
Methanol, 0.01 M HCl	*b*	*b*	−55	−345	+1420	−1095

[a] Parameters based on the dispersion data in the 313–578 mμ region. [b] Dispersion is anomalous (*i.e.*, the $[\alpha]\lambda\lambda^2$ *vs.* $[\alpha]\lambda$ plot is not linear).

Upon denaturation in 8 M urea these changed to −900° and +60°, respectively.[4] Similar observations were made by von Hippel and Wong (1963) on ichthyocol collagen.

TABLE VII: Estimates of Helix Content of the Caseins in Helix-Promoting Solvents.[a]

Protein and Solvent	% Helix		
	Moffitt Eq (5)	Shecter and Blout Eq (9)	Shecter and Blout Eq (10)
α_{s1}-Casein C			
Ethylene glycol	22	28	30
Methanol, 0.01 M HCl	55	59[b]	52[b]
2-Chloroethanol	48	51	48
β-Casein A			
2-Chloroethanol	36	39	41
κ-Casein			
2-Chloroethanol, 0.01 M HCl	56	55	61
Whole casein I			
2-Chloroethanol	59	57	63

[a] Estimates based on data of Tables V and VI. [b] The methanol values are based on eq (6) and (7). These equations are found to be more appropriate for more polar, hydrogen-bonding solvents (Schecter and Blout, 1964,b).

[4] In addition to having a positive Moffitt b_0 parameter, collagen and polyproline II also exhibit strongly negative Cotton effects, with minima at 210–220 mμ (*e.g.*, $[R']_{208} \sim -35{,}000$ for calfskin collagen (Blout *et al.*, 1963). Based on b_0, polyproline II-like regions in proteins would thus lead to fortuitously low estimates of helix content, while calculations based on the trough value of the 225-mμ Cotton effect would lead to high estimates of helix content. The close agreement between these two estimates of helix content for the caseins (Table II) suggests that such regions must be largely absent in the case of these proteins at room temperature.

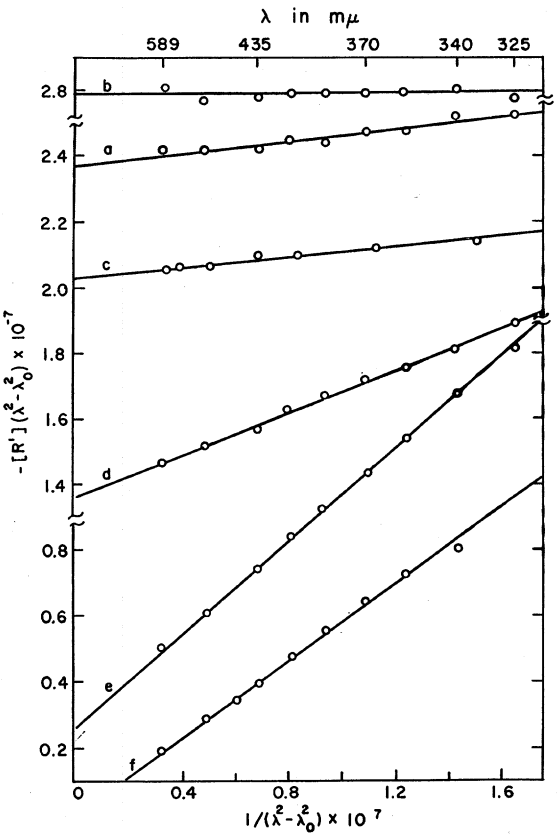

FIGURE 3: Moffitt–Yang plots of α_{s1}-casein C in aqueous and organic solvents. Curve a, water, 0.03 M Cl^-, pH 7.4; curve b, 8 M urea, 0.03 M Cl^-, pH 3.3; curve c, 97% formic acid; curve d, ethylene glycol; curve e, methanol, 0.01 M HCl; curve f, 2-chloroethanol. Lines were computed by the method of least squares.

The effect of a second random coil promoting solvent, formic acid, on the rotatory properties of α_{s1}-casein is also relatively small. On the other hand, the solvents ethylene glycol, 2-chloroethanol, and methanol have a pronounced effect on the rotatory properties of

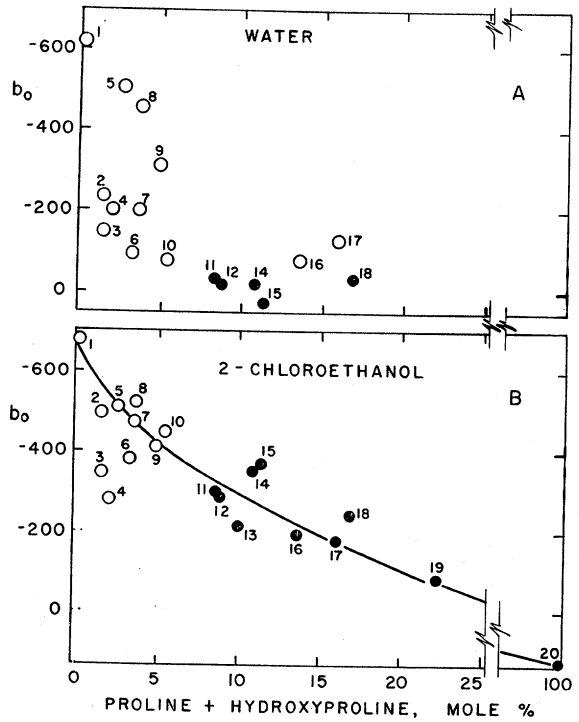

FIGURE 4: Variation of the Moffitt b_0 parameter as a function of proline and hydroxyproline content of proteins; (A) in aqueous solution; (B) in 2-chloroethanol. Numbers designate the following proteins and polypeptides: 1, tropomyosin; 2, α-lactalbumin; 3, lysozyme; 4, insulin; 5, myoglobin and globin M (in 2-chloroethanol); 6, ribonuclease; 7, ovalbumin; 8, hemoglobin and globin H (in 2-chloroethanol); 9, bovine serum albumin; 10, β-lactoglobulin; 11, α_{s1}-casein C; 12, α_{s1}-casein B; 13, zein; 14, κ-casein; 15, whole casein I; 16, glutenin; 17, whole gluten; 18, β-casein A; 19, calfskin collagen; 20, poly-L-proline. Filled circles refer to data of Tables I, V, and VI; open circles refer to data taken from Urnes and Doty (1961), Beychok and Blout (1961), Herskovits and Mescanti (1965), and Wu and Cluskey (1965).

the caseins (Tables V-VII and Figure 3). Judging by the changes in the Moffitt and the Schechter and Blout parameters, it appears that the helical content of the caseins can be increased to as much as 50–60% (Tables II and VII).

Effects of Variations in the Chemical Composition of the Caseins. There are three known genetic variants of both α_{s1}- and β-caseins (Thompson and Kiddy, 1964; Thompson and Pepper, 1964) and recently genetic polymorphism has also been found in κ-casein (Woychik, 1964). The three polymorphs of α_{s1}- and β-caseins are designated A, B, and C (Thompson and Kiddy, 1964). As in the case of the β-lactoglobulins (Herskovits *et al.*, 1964; Townend *et al.*, 1964) genetic polymorphism, that is the replacement of substitution of individual amino acids as a result of genetic mutations, has little or no effect on their rotatory dispersion properties and, therefore, on their secondary structure This is shown by the data of Table I, where the rotatory dispersion properties of α_{s1}-casein B and C and β-casein A and B are compared.

Effect of Proline Content. Figure 4 presents a correlation between the Moffitt b_0 parameter, which reflects the helical content of proteins, and the proline and hydroxyproline content of various proteins, including the caseins, in aqueous media and in 2-chloroethanol. The data represented by open circles were taken from the literature, while the filled circles represent data of this study (Table I and V). The data obtained in aqueous media on the caseins are in agreement with the conclusion of Szent-György and Cohen (1957) concerning the essentially nonhelical character of proline-rich proteins. As is expected, even in the strongly helix-promoting solvent, 2-chloroethanol, the helical content of the caseins as well as the other proline-rich proteins, zein, gluten, and glutenin, is lower than that of proteins that contain less proline.

Discussion

It has been recognized for some time that the proline ring cannot be accommodated into the α-helical fold in proteins. Szent-György and Cohen (1957) have concluded, from optical rotatory dispersion studies on various myosins and fibrinogen, that the disruptive influence of proline on the polypeptide chain is such that with about 8% or more proline statistically distributed through the protein the polypeptide backbone will be deformed into a random coil. The proline content of the caseins is 8.5–16.8 mole % (Table V); furthermore, other amino acids (valine, isoleucine, serine, threonine, and the cystine contained in κ-casein (Jollès *et al.*, 1962; Swaisgood and Brunner, 1963)) may also interfere with helix formation (Blout *et al.*, 1960; Davies, 1964). It is then not surprising that the caseins have little α-helical organization (Table II).[1]

The effect of temperature and organic solvents on the optical rotatory properties of the casein is consistent with the idea that these proteins have a disorganized structure, usually encountered in the case of denatured proteins. One would expect on the basis of Schellman's (1958a,b) studies on denatured proteins that the rotatory properties of proteins with little structural organization should respond to changes in temperature (Table IV). Moreover, as is also expected, urea and formic acid, two random coil forming solvents, have relatively little effect on the rotatory properties of the caseins (Table II). On the other hand, structure-forming solvents such as 2-chloroethanol and acidic methanol are found to promote a fairly high degree of α-helical folding in these proteins (Tables V, VI, and VII). The fact that the caseins in their native state are readily digested by proteolytic enzymes (Northrop *et al.*, 1948; Nomoto *et al.*, 1960) is also consistent with the lack of extensive secondary and tertiary structural organization of these proteins. Linderstrøm-Lang (1952) some time ago made the observation that globular proteins often are partially or even totally resistant

to enzymatic attack in their native state. The same proteins are readily digested by these enzymes upon denaturation.

Waugh (1958) has suggested on the basis of hydrodynamic and molecular weight information that the three main casein components, α_s-, β-, and κ-casein, are rod-like macromolecules with lengths of 150–215 A and approximate diameters of 16 A. The fact that κ-casein could interact with four times its weight of α_s-casein (Waugh and von Hippel, 1956) has led to the notion (Waugh, 1958) that the probable structure of the α_s–κ-casein complex was a rod-like side-to-side aggregate consisting of four peripherally located α_s-casein molecules and one centrally located, but slightly protruding κ-casein molecule. More recently, however, Zittle *et al.* (1962) and Waugh and Noble (1965) have found that α_s–κ-casein complexes may actually be stabilized at weight ratios far in excess of 1:4, suggesting that Waugh's earlier model of casein complexes may be an oversimplification (see also Noble and Waugh, 1965).

While the rotatory dispersion data obtained on whole casein (Tables I and II) are not actually inconsistent with Waugh's model of the α–κ-casein complex, from what is known about the effect of intermolecular association of proteins (Schellman, 1958a,b; Urnes and Doty, 1961; Herskovits *et al.*, 1964) one would be led to believe that the formation of ordered rod-like aggregates from random chains of the individual casein components should cause a greater change in the optical rotatory properties than is actually observed[5] (Table II and Figures 1 and 2). These observations, together with the recent discovery (Beeby, 1963; Swaisgood and Brunner, 1963; Swaisgood *et al.*, 1964) that κ-casein consists of two or three disulfide-bond-linked polypeptide chains and has a molecular weight of about 50,000 (Waugh assumed that κ-casein consists of a single polypeptide chain having a molecular weight of 16,000), will require a revision of Waugh's earlier model of the basic subunit of casein micelles in solution. Recently Shimmin and Hill (1964) have advanced, on the basis of electron microscopic studies, an approximately spherical α_s–κ–β-casein complex of about 300,000 mol wt as a possible model for the basic subunit of casein micelles.

[5] Assuming that upon monomer formation the a_0 values of α_{s1}- and κ-casein would change by about the same amount as β-casein (20%; Table IV), the a_0 value of a hypothetical mixture of the major casein components (55% α_{s1}-, 30% β-, and 15% κ-casein; Lindquist, 1963) in their monomeric state would be equal to −735. In comparison, the average a_0 value of whole casein aggregates is −750 (Table I). The a_0 values of most denatured proteins and polypeptides in the random-coil form range from −500 to −800 (Urnes and Doty, 1961). Differences between the other rotatory dispersion parameters (λ_c, b_0, $A(\alpha,\rho)_{193}$, $A(\alpha,\rho)_{225}$, and $[R']_{233}$) of the individual casein components and whole casein are also relatively insignificant (Table I and IV), suggesting no obvious structural differences between whole casein aggregates and their constituent casin components.

Acknowledgment

The author is grateful to Drs. M. Noelken, G. C. Nutting and M. P. Thompson for valuable discussions and criticism of the manuscript. He also wishes to thank Dr. W. Gaffield for performing the rotatory dispersion measurements in the ultraviolet region for him, Dr. Y. V. Wu for a prepublication copy of a manuscript, Mr. T. F. Kumosinski for the computer calculations and Miss L. Mescanti for expert technical assistance.

References

Beeby, R. (1963), *J. Dairy Res. 30*, 77.
Beychok, S., and Blout, E. R. (1961), *J. Mol. Biol. 3*, 769.
Blout, E. R., Carver, J. P., and Gross, J. (1963), *J. Am. Chem. Soc. 85*, 644.
Blout, E. R., deLozé, C., Bloom, S. M., and Fasman, G. D. (1960), *J. Am. Chem. Soc. 82*, 3787.
Blout, E. R., Schmier, I., and Simmons, N. S. (1962), *J. Am. Chem. Soc. 84*, 3193.
Breslow, E., Beychok, S., Hardman, K. D., and Gurd, F. R. N. (1965), *J. Biol. Chem. 240*, 304.
Davies, D. R. (1964), *J. Mol. Biol. 9*, 605.
Gordon, W. G., and Whittier, E. O. (1965), *in* Fundamentals of Dairy Chemistry, Webb, B. H., and Johnson, A. H., Ed., Westport, Conn., Avi Publishing Co., p 54.
Harrington, W. F., and Sela, M. (1958), *Biochim. Biophys. Acta 27*, 24.
Harrison, S. C., and Blout, E. R. (1965), *J. Biol. Chem. 240*, 299.
Herskovits, T. T. (1964), Abstracts, 146th National Meeting of the American Chemical Society, Denver, Colo., Jan, p 22A.
Herskovits, T. T. (1965), *J. Biol. Chem. 240*, 628.
Herskovits, T. T., and Mescanti, L. (1965), *J. Biol. Chem. 240*, 639.
Herskovits, T. T., Townend, R., and Timasheff, S. N. (1964), *J. Am. Chem. Soc. 86*, 4445.
Hipp, N. J., Groves, M. L., Custer, J. H., and McMeekin, T. L. (1952), *J. Dairy Sci. 35*, 272.
Jirgensons, B. (1958a), *Arch. Biochem. Biophys. 74*, 57.
Jirgensons, B. (1958b), *Arch. Biochem. Biophys. 74*, 70.
Jollès, P., Alais, C., and Jollès, J. (1962), *Arch. Biochem. Biophys. 98*, 56.
Kresheck, G. C. (1965), *Acta Chem. Scand. 19*, 375.
Kresheck, G. C., van Winkle, Q., and Gould, I. A. (1964), *J. Dairy Sci. 47*, 117.
Linderstrøm-Lang, K. U. (1952), Proteins and Enzymes, Stanford, Calif., Stanford Univ. Press, p 53.
Lindqvist, B. (1963), *Dairy Sci. Abstr. 25*, 257.
Noble, R. W., Jr., and Waugh, D. F. (1965), *J. Am. Chem. Soc. 87*, 2236.
Nomoto, M., Narahashi, Y., and Murakami, M. (1960), *J. Biochem. (Tokyo) 48*, 593.
Northrop, J. H., Kunitz, M., and Herriott, R. M. (1948), Crystalline Enzymes, 2nd ed, New York, N. Y., Columbia Univ. Press, p 120.

Payens, T. A. J., and van Markwijk, B. W. (1963), *Biochim. Biophys. Acta 71*, 517.
Schellman, J. A. (1958a), *Comp. Rend. Lab. Carlsberg Sér. Chim. 30*, 395.
Schellman, J. A. (1958b), *Comp. Rend. Lab. Carlsberg Sér. Chim. 30*, 439.
Shechter, E., and Blout, E. R. (1964a), *Proc. Natl. Acad. Sci. U. S. 51*, 695.
Shechter, E., and Blout, E. R. (1964b), *Proc. Natl. Acad. Sci. U. S. 51*, 794.
Shechter, E., Carver, J. P., and Blout, E. R. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 1029.
Shimmin, P. D., and Hill, R. D. (1964), *J. Dairy Res. 31*, 121.
Simmons, N. S., and Blout, E. R. (1960), *Biophys. J. 1*, 55.
Simmons, N. S., Cohn, C., Szent-Györgyi, A. G., Wetlaufer, D. B., and Blout, E. R. (1961), *J. Am. Chem. Soc. 83*, 4766.
Sullivan, R. A., Fitzpatrick, M. M., Stanton, E. K., Annino, R., Kissel, G., and Palermiti, F. (1955), *Arch. Biochem. Biophys. 55*, 455.
Swaisgood, H. E., and Brunner, J. R. (1963), *Biochem. Biophys. Res. Commun. 12*, 148.
Swaisgood, H. E., Brunner, J. R., and Lillevik, H. A. (1964), *Biochemistry 3*, 1616.
Szent-Györgyi, A. G., and Cohen, C. (1957), *Science 126*, 697.
Thompson, M. P., and Kiddy, C. A. (1964), *J. Dairy Sci. 47*, 626.
Thompson, M. P., and Pepper, L. (1964), *J. Dairy Sci. 47*, 633.
Timasheff, S. N. (1964), *in* Symposium on Foods: Proteins and Their Reactions, Schultz, H. W., and Anglemier, A. F., Ed., Westport, Conn., Avi Publishing Co., p 179.
Townend, R., Herskovits, T. T., Swaisgood, H. E., and Timasheff, S. N. (1964), *J. Biol. Chem. 239*, 4196.
Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.
von Hippel, P. H., and Wong, K.-Y. (1963), *Biochemistry 2*, 1387.
Waugh, D. F. (1958), *Discussions Faraday Soc. 25*, 186.
Waugh, D. F., and Noble, R. W., Jr. (1965), *J. Am. Chem. Soc. 87*, 2246.
Waugh, D. F., and von Hippel, P. H. (1956), *J. Am. Chem. Soc. 78*, 4576.
Woychik, J. H. (1964), *Biochem. Biophys. Res. Commun. 16*, 267.
Wu, Y. V., and Cluskey, J. E. (1965), *Arch. Biochem. Biophys. 112*, 32.
Yang, J. T. (1961), *Tetrahedron 13*, 143.
Yang, J. T. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 438.
Yang, J. T., and Samejima, T. (1963), *J. Biol. Chem. 238*, 3262.
Zittle, C. A., and Custer, J. H. (1963), *J. Dairy Sci. 46*, 1183.
Zittle, C. A., Thompson, M. P., Custer, J. H., and Cerbulis, J. (1962), *J. Dairy Sci. 45*, 807.